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CK19 and OV6 Expressions in the Liver of 2-AAF/CCl_4 Rat Model

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Abstract

BACKGROUND: Liver regeneration on a chronic liver injury with rat model research is urgent to study the pathology of human chronic diseases. 2-Acetylaminofluorene (2-AAF) and carbon tetrachloride (CCl4) can be applied to study about liver regeneration by liver stem cells, known as oval cells. The use of 2-AAF and CCl4 (2-AAF/CCl4) to induce chronic liver injury can lead severe hepatocyte damages and extensive fibrosis.

AIM: This study aims to observe the OV6 and CK19 expression in the liver of 2AAF/CCl4 rat model.

METHODS: In this research, a high dose of 2-AAF (10 mg/kg) was applied and combined repeatedly with CCl4 (2 ml/kg) for 12 weeks. An immunohistochemistry (IHC) procedure using OV6 and CK19 antibodies was also applied to examine the regeneration of oval cells. Both antibodies expressions were then examined semi-quantitatively according to the expressions percentage of each sample based on the liver zone. We have observed the OV6 and CK19 expressions in every zone, including in portae hepatis area.

RESULTS: The results showed OV6 and CK19 with the highest expression in the Zone I. A significant difference between OV6 and CK19 expressions was revealed in healthy (control group) and 2-AAF/CCl4 groups (both indicating $p = 0.045$). Moreover, a ductular reaction was also found in Zone I and II of the 2-AAF/CCl4.

CONCLUSION: We conclude that 2-AAF/CCl4-induced chronic rat liver injury model could be utilized to investigate the oval cells with OV6 and CK19 expression.

Introduction

A chronic liver injury rat model research is needed to mimic the pathology of chronic liver diseases in human. Rats induced by 2-Acetylaminofluorene (2-AAF) combined with carbon tetrachloride (CCl4) can provide proportional conditions to obtain a model of liver injury. Liem *et al.* (2020) asserted that daily induction of a high dose of 2-AAF (10 mg/kg) for 12 weeks combined with repeated application of CCl4 (2 ml/kg) for 12 weeks can cause severe and extensive hepatocytes damages and fibrosis [1]. This research makes advanced observation of endogenous liver stem cells regeneration from paraffin block-stored rat liver tissue assessment.

Induction of chronic liver injury with 2-AAF and CCl4 will cause liver damage, impaired hepatocyte proliferation, and the formation of fibrotic tissue. 2-AAF, an aromatic amine is metabolized in the liver to N-hydroxy-2-AAF, is toxic to the proliferative ability of hepatocytes. Meanwhile, CCl4 has ability to induce hepatocyte necrosis, especially in the centrilobular region, and cause damage to the sinusoids at the central vein area. The damage can be developed by the metabolized CCl4 substance, which are trichloromethyl (CCl3) and chlorine, so the hepatocytes will produce free radicals that cause direct hepatocyte apoptosis and necrosis [2]. This condition activates pro-inflammatory cells that evoke further hepatocyte death together with fibrotic tissue construction. The pathological conditions, liver massive cell death and fibrotic tissue formation, are commonly found in human chronic liver diseases [2].

Chronic liver injury has the potential to cause irreversible tissue changes due to damage parenchymal liver cells, namely, hepatocytes and cholangiocytes [3]. Chronic injury in hepatocytes can also interfere the production and secretion of bilirubin, resulting in accumulation of bilirubin in the liver (cholestasis). In addition to the hepatocytes disruption, chronic liver injury generates the conversion of liver connective tissue to fibrotic tissue. This pathological condition may impair liver blood flow and disturbing hepatic portal vein blood pressure. Consequently, many organs that drain

the blood through the veins encountered blood flow resistance. In addition, the continuous fibrotic formation of connective tissue can lead to end-stage fibrotic liver which is also known as liver cirrhosis [4]. Tsochatzis *et al.* (2014) concluded that impaired liver function due to cirrhosis will imply overall impaired liver function. In addition, cirrhosis itself is a threat to be a malignant hepatocellular carcinoma [5].

More importantly, chronic liver injury may evoke regeneration of endogenous liver stem cells. In rats, the regeneration by endogenous stem cells (oval cells) will be activated after the impaired ability of liver regeneration by hepatocytes. Further, to study oval cell regeneration, OV6 and cytokeratin 19 (CK19) expressions have been widely used as markers. OV6 is a protein marker that can generally identify epitope from CK 14 and 19. On the other hand, CK19 is well known as a specific marker of biliary/progenitor and tumor stem cells [6]. In addition, CK19 constitutes an intermediate filament with a molecular weight of about 40 kDa. During embryonic development, OV6 and CK19 are detected as progenitor cells of primitive liver in 4–10 weeks of pregnancy. As fetal liver grows, these bipotential progenitor cells can differentiate to be hepatocytes and cholangiocytes. Afterward, CK19 and OV6 expression will be unrevealed in adult hepatocytes while CK19 persistently is expressed in cholangiocytes. This points out that both expressions can be used as liver regeneration markers by endogenous liver stem cells in a chronic liver injury [6].

With the chronic liver injury model, induced by 2AAF (10 mg/kg) in combination with repeated CCl4 (2 ml/kg) for 12 weeks, liver parenchymal cells (hepatocyte) will be severely damaged, and extensive fibrotic tissue will replace the native liver connective tissue. Since the model of chronic injury would impair regeneration by hepatocytes, we do not yet know the pattern of the oval cells when oval cell regeneration occurs. Hence, a study was conducted to see how oval cells express OV6 and CK19 in chronic liver injury with extensive fibrosis. This research was conducted by immunohistochemistry (IHC) staining to study the expression of these proteins based on the liver zonation. We expect that the results can be applied to further research on oval cell regeneration in liver injury and provide a perspective for the basics of liver damage treatment through liver stem cell regeneration.

Methods

This current research employed an experimental study design on male Wistar rats (*Rattus norvegicus*) aged 8 weeks and weighed 150–200 g. The rats were classified into two groups, the control (Group 1) and 2-AAF/CCl4 induced (Group 2). Each group contained six rats. The same grouping scheme was also applied to the research of Liem *et al.* [1], as a standard of 2-AAF/CCl4-induced liver injury model. The research, in addition, had acquired the approval letter issued by the Committee of Health Research Ethics of Medical Faculty, Universitas Indonesia, by the reference number of 1040/UN2.F1/ETIK/2018.

For the current study, liver tissue, stored in paraffin blocks, was used as the main material. For liver injury induction, the 2-AAF and CCl4 were applied and obtained from Sigma-Aldrich® (Darmstadt, Germany) and Merck® (Munich, Germany). IHC staining methods were performed using primary antibodies in OV6 (Mab2020, R&D System) and CK19 (Ab220193, R&D System), Histofine secondary antibodies® Universal Immuno-enzyme Polymer), 10% Normal Horse Serum, diaminobenzidine tetrahydrochloride as chromogenic substance, 3% H_2O_2 as an endogenous peroxide inhibitor agent, phosphate buffer solution with pH 7.4; 6.0 pH sodium citrate, and 9.0 pH Tris-EDTA solution. For counterstain, we used hematoxylin Mayer (HE) and lithium carbonate solution.

Samples of OV6 and CK19 IHC were obtained from digital imagery of Zones 0 (hepatic portae vein), I, II, and III. Five images were captured for each zone digitally with a 400× zoom magnification. The analysis was conducted by assessing the percentage of OV6 and CK19 expressions using ImageJ IHC Profiler® software. The average expression generated by the software was converted into a semi-quantitative calculation of optical density through the basic formula as followed: ([positive percentage “strong” × 4] + [positive percentage “medium” × 3] + [positive percentage “weak” × 2] + [negative percentage × 1])/100. The distribution of OV6 and CK19 expression distribution in each zone was assessed and presented descriptively. Finally, the data obtained were statistically processed by the Statistical Product and Service Solution (SPSS®) version 20.0.

Results

This research was conducted by IHC staining to study the expression of these proteins based on the liver zonation. We expect that the results can be applied to further research on oval cell regeneration in liver injury and provide a perspective for the basics of liver damage treatment through liver stem cell regeneration. The results of the OV6 IHC examination were observed in areas representing the liver zonation system in both the control and the 2-AAF/CCl4 induction groups (Figure 1a-h). The zonation included Zone I (periportal area), Zone II (area between periportal and pericentral area), and Zone III (pericentral area, specifically near centralis vein). Meanwhile, Zone 0

(Figure 1a) constituted hepatic portal area, in which the observation of OV6-positive oval cells excluded the oval cells in Zone I. Further, a ductular reaction (DR) was only vibrant in Zones I and II.

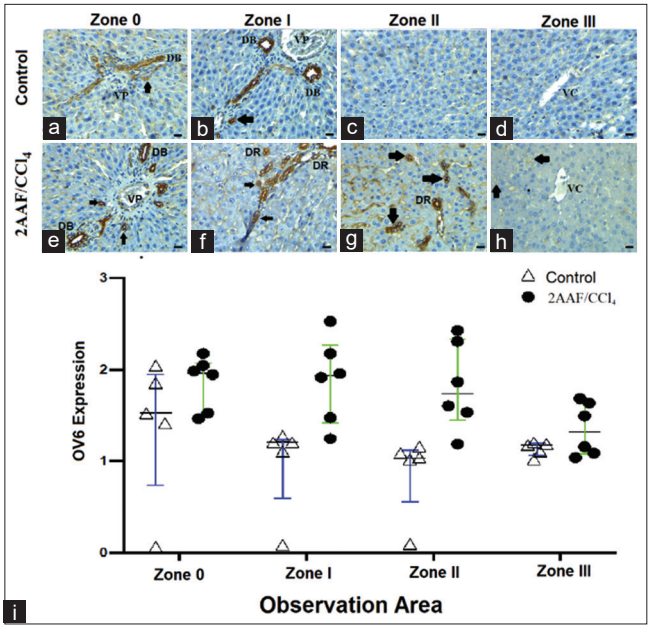

Figure 1: The IHC examination overview of OV6 expression on 400× zoom magnification (a-h) and graph (i) of both the control and induction 2-AAF/CCl4 groups. The observation areas were Zone 0 (a and e), Zone I (b and f), Zone II (c and g), and Zone III (d and h). VP: Vein portae, VC: Vein centralis, DB: Ductus biliaris, DR: Ductular reaction, black arrow: OV6-positive oval cell, black bar: 10 μm scale

Oval cells were observed to be positive for OV6 antibodies in the 2-AAF/CCl4-induced group (Group 2) compared to the control group (Group 1). In the control group, OV6-positive oval cells were confined to the border areas of portal area and Zone I (Figure 1a and b). No DR was observed in all zones of Group 1. DR in Group 2 was found in Zones 0, I, and II (Figure 1e-g). In the control group, the cholangiocytes of ductus biliaris (DB) appeared to express OV6 (Figure 1a and b). In the control group, the cholangiocytes of ductus biliaris (DB) appeared to express OV6 (Figure 1a and b), in contrast, in Group 2 gave many OV6-positive oval cells appearance in all zones (Figure 1e-Ih).

The OV6 expression in these two groups was assessed semi-quantitatively by its zone and presented as a graphic at Figure 1i. The 2-AAF/CCl4 group (Group 2) showed higher OV6 expression than the control group. Group 2 indicated high expression patterns in Zones 0, I, and II, as well as the lowest expressions in Zone III. In contrast, the OV6 expression pattern in the control group (Group 1) showed high expression in Zone 0, while the expressions of OV6 Zones I, II, and III were relatively the same and lower than those of Zone 0. The 2-AAF/CCl4 induction group also showed significant differences in OV6 expression in Zones I ($p = 0.045$) and II ($p = 0.006$) compared to the control group. DR appearance was also found in the 2-AAF/CCl4 induction group which was accompanied by an increase of OV6 expression in each zone. This increase is predominantly seen in Zone I (mean = 1869).

As in the OV6 expression examination results, the control group (Group 1) showed CK19-positive oval cells and was limited to the portal hepatis border with Zone I (Figure 2a and d). DR was also not found in all zones. Meanwhile, oval cells with positive CK19 expression in the 2-AAF/CCl4 induction group (Group 2) could possibly be observed in all zones, especially in Zones I and II (Figure 2f and g). Zone III could be found fewer number of oval cells with CK19 expression. Observations of DR were also found in Zones I and II (Figure 2e and f), but were not found in Zone III (Figure 2c).

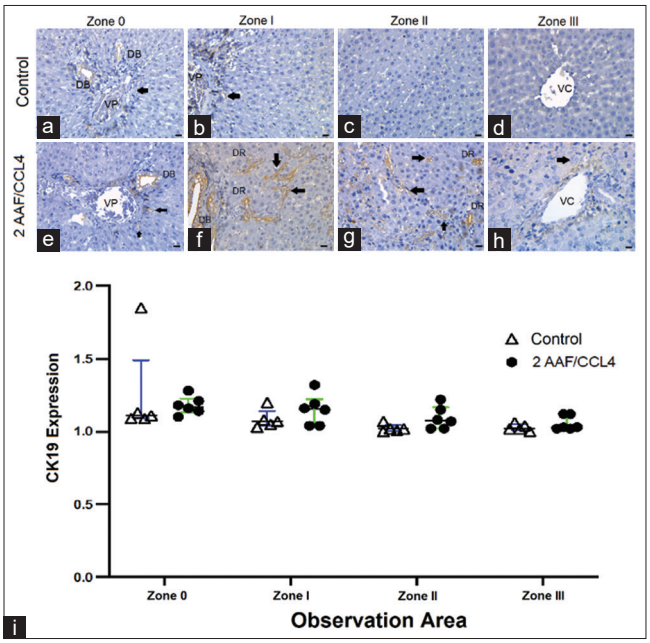

Figure 2: The IHC examination overview of CK19 expression on 400× zoom magnification (a-h) and graph (i) of both the control and induction 2-AAF/CCl4 groups. The observation areas were Zone 0 (a and e), Zone I (b and f), Zone II (c and g), and Zone III (d and h). Notes: VP: V. portae, VC: V. centralis, DB: Ductus biliaris, DR: Ductular reaction, black arrow: OV6-positive oval cell, black bar: 10 μm scale

The CK19 expression assessment on liver tissues of rat was carried out semi-quantitatively in each of zones. It was indicated that the induced (Group 2) expressed CK19 more than the control (Group 1) (Figure 2i). In Zone 0, the control group had higher CK19 expression (mean 1.1786) than the 2-AAF/CCl4 group (mean = 1.2426). Furthermore, Zones I, II, and III of the induction group 2-AAF/CCl4 tended to have higher CK19 expression than the control group. CK19 was more expressed in the induction group 2-AAF/CCl4 (average = 1.1197) than the control group (average = 1.1098). Statistical analysis showed no significant difference in all zones between the control and 2-AAF/CCl4 groups.

Discussion

Our research revealed the activation of oval cells when hepatocytes were massively damaged and lost their proliferation ability. This animal model with chronic liver injury approaches a pathological state in chronic diseases of the human liver, particularly by non-alcoholic causes such as viral hepatitis and liver intoxication [7], [8]. Rats model, injured by 2-AAF/CCl4, can cause massive hepatocyte damage and fibrotic tissue development. Research by Liem *et al.* [1] using the same group of rats showed liver damage due to necrosis and apoptosis, as well as the formation of fibrosis tissue. The main regenerating ability of the remaining hepatocyte liver was inhibited, so regeneration by oval cells was needed [8], [9]. Another method that is widely used to investigate oval cell regeneration is by cutting 2/3 of the liver (partial hepatectomy). Unfortunately, the mechanism of liver tissue regeneration is not only through proliferation but also hyperplasia of the remained hepatocyte with the remarkably increased physiological abilities [10], [11]. This is the virtue of enhancing 2-AAF as part of liver injury procedure to inhibit hepatocyte proliferation. The result will be apparent secondary regeneration mechanism by oval cells [7]. However, Dusabineza *et al.* [12] research proved that only a few oval cells contributed to hepatocytes regeneration due to a partial hepatectomy followed by 2-AAF [12].

Chronic liver injury induced by a combination of CCl4 and 2-AAF evoked the activation of oval cell regeneration by inhibiting regeneration by hepatocytes [11]. CCl4 induces hepatocyte injury from the centrilobular zone as well as pericentral sinusoid damage through its metabolic results, trichloromethyl (CCl3-). The metabolism by hepatocyte itself will produce free radicals within the hepatocyte itself and lead to the induction of apoptosis and necrosis. In accordance with Hasan and Lettuce (2015) models, the liver injury with the use of 2-AAF/CCl4 can uplift the expression of caspase 3 which indicates an increase in hepatocyte apoptosis. It was supported by Liem *et al.* (2021) research, confirming that the injury model could arise an immense liver injury with fibrosis. In addition [13], CCl4 also served to evoke hepatocyte death through lipid peroxide in hepatocyte membranes and to upsurge mitochondrial permeability [14]. Furthermore, the administration of CCl4 itself generally induces differentiation of oval cells through inflammatory cells as the impact of CCl4 to liver tissue fibroblasts [15]. CCl4 is also well known to damage the liver extracellular tissue which stimulates the formation of fibrosis tissue through the Stellate cells activity [16], [17]. The modification of extracellular matrixes due to fibrosis evoked a niche alteration of oval cells, which became one of the determinant factors of oval cell activation and proliferation. Meanwhile, the role of an aromatic amine of 2-AAF which is metabolized by cytochrome P450 liver into N-hydroxy-2-AAF will lead damage hepatocytes DNA structure. The impact is a failure for hepatocytes to undergo the proliferation [18], [19], [20], [21], [22]. The both mechanisms of 2-AAF/CCl4 combination will inhibit hepatocyte regeneration and activate oval cell response to do regeneration.

Our results showed the oval cells regeneration activation by proliferation and migration after 12 weeks of chronic injury 2-AAF/CCl4 induction. Thus, as expected, the expressions of OV6 and CK19 were observed in oval cells. Using liver zone approached observations, the direction of oval cells migration could be notably seen from periportal to pericentral area. This observation is in accordance with the results of the Goradel *et al.* study which states that the bipotential regenerating oval cells have the capability to infiltrate through the liver parenchymal plate when differentiated into hepatocytes and cholangiocytes [23], [24], [25], [26].

The time oval cells needed to regenerate varies and aligns with the rate of their proliferation and expansion. Roskams *et al.* used choline-deficient acetylaminofluorene as material for chronic rat liver injury induction to examine the course of oval cell regeneration and marked the oval cells with antibodies OV6 and CK19 [8]. After the induction was terminated, observations on the 14th day showed significant proliferation and expansion of oval cells compared to day 7. Observations on day 7 showed that the proliferation of oval cells was limited to Zone I (periportal areas). The observations with antibody markers against CK19 also indicated a significant appearance of Dr. Dusabineza *et al.* [12] used the 2-AAF/PH model and compare oval cell regeneration on days 7, 10, and 14 days after chronic injury induction. Referring to the observations, CK19-positive oval cells on days 10 and 14 showed more proliferation and expansion from Zone I to Zone III, predominantly in Zone II compared to observations on day 7. Abdellatif *et al.* [18] used the 2-AAF/CCl4 induction for developed chronic injury rats model to find the therapeutic effect of mesenchymal stem cells of human umbilical cord blood origin. Examination at 9 days after the induction of chronic injury, oval cells showed proliferation, both solitary and forming the DR structure. Oval cell expansion was also observed to be far from Zone I to Zone III. Chen *et al.* [19] also used the CCl4/2-AAF induction model to induce chronic liver injury to investigate the role of the non-canonical Wnt pathway. Twelve weeks were considerably sufficient for induction time with CCl4/2-AAF to obtain massive oval cell proliferation and expansion from Zone I to Zone III. The emergence of DR could be also observed up to 14 weeks after CCl4/2-AAF induction. Expansion of oval cells to Zone III can even be obviously seen as a connecting bridge between the portal and the pericentral area in the 2nd week or after. Thus, it can be concluded that the rat model of chronic liver injury by 2AAF/CCl4 may lead to oval cell regeneration, at least, since the 2nd week after the induction.

Conclusion

Liver injury after 2AAF/CCl4 induction for 12 weeks, in addition caused extensive liver damage with fibrosis. A chronic liver injury of rat induced with 2-AAF/CCl4 for 12 weeks is effective to express OV6 and CK19 as the main marker of activated oval cell regeneration. The CK19 expression pattern was observed to be consistent with OV6 from periportal to pericentral zone. This indicates the direction of expansion of oval cell regeneration from its niche, which is located in the portal area to penetrate the heart parenchyma toward the central vein.

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